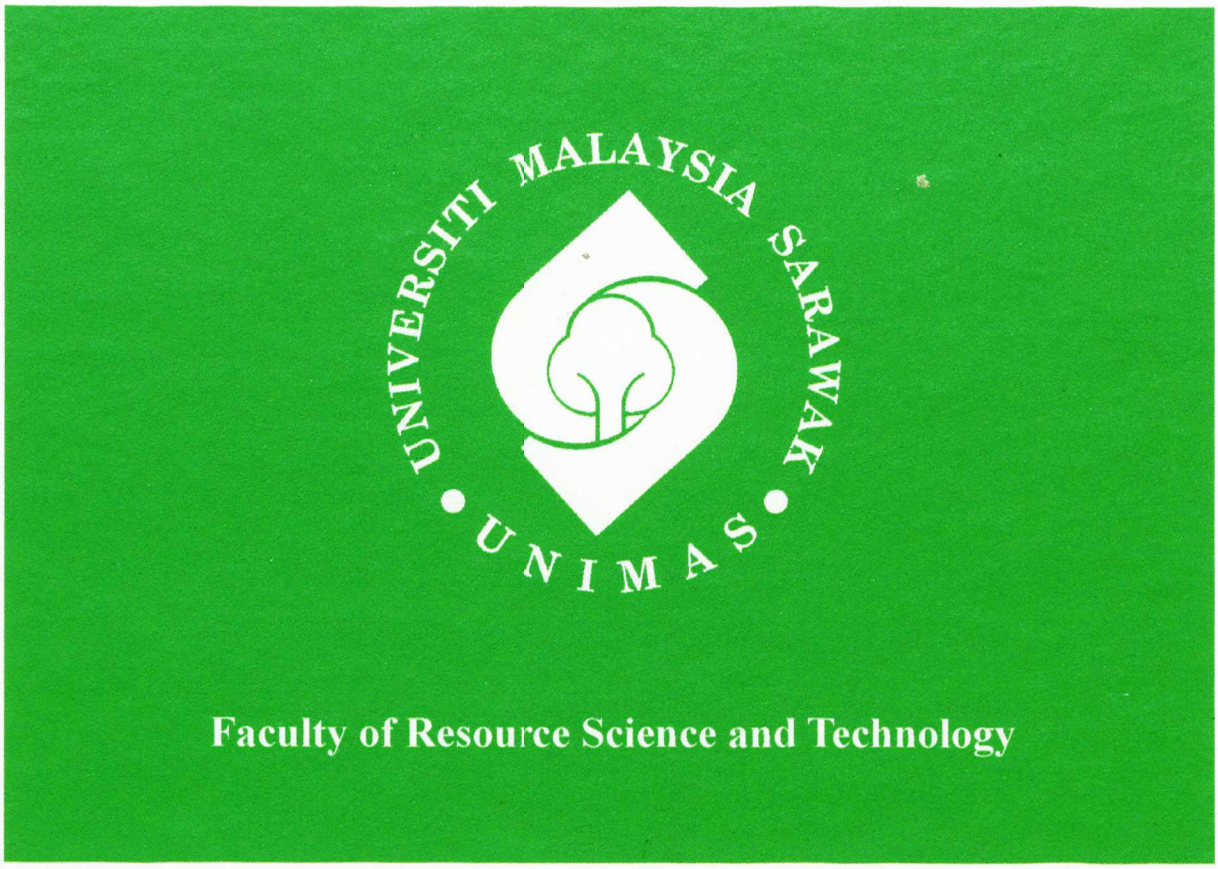

Assessment of the Effectiveness of Chlorine Solution as Sterilization Method on *Burkholderia pseudomallei* and *Burkholderia* Species in Laboratory

Angeline Michael (50398)

Bachelors of Science with Honours
(Resource Biotechnology)
2018

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ASSESSMENT OF THE EFFECTIVENESS OF CHLORINE SOLUTION AS STERILIZATION METHOD ON *BURKHOLDERIA PSEUDOMALLEI* AND *BURKHOLDERIA* SPECIES IN LABORATORY

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April 2018

UNIVERSITI MALAYSIA SARAWAK

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Final Year Project Report ☑

Masters ☐

PhD ☐

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Assessment of the Effectiveness of Chlorine Solution as Sterilization Method on *Burkholderia pseudomallei* and *Burkholderia* Species in Laboratory

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ABSTRACT

In this study, the method of sterilizing *Burkholderia pseudomallei* and *Burkholderia* species were using chlorine solution, which is known as one of the cheapest disinfectant for wide scope of microorganisms. The effectiveness of chlorine sterilization was evaluated at different concentrations of chlorine with variating contact time and shelf-lives of the chlorine solution. Results indicated that chlorine concentration is effective in the sterilization of *Burkholderia* species and *Burkholderia pseudomallei* in the range of at least 15 mg/L. In addition, the effectiveness of the chlorine solution is maintained if used within 2 days of preparation with no significant effect out from the aspect of the contact time. Hence, effectiveness of chlorine solution to sterilize *B. pseudomallei* and *Burkholderia* species were in chlorine concentration of 15 mg/L made within 48 hours from preparation date.

Key words: *Burkholderia* species, sterilization, chlorine solution

ABSTRAK

Dalam kajian ini, kaedah pensterilan Burkholderia pseudomallei dan spesies Burkholderia ialah penggunaan larutan klorin, yang turut dikenali sebagai salah satu pembasmi kuman yang murah bagi skop mikroorganisma yang luas. Keberkesanan pensterilan klorin dinilai mengikut kepekatan klorin yang berbeza dengan pelbagai masa perhubungan dan jangka hayat larutan klorin. Keputusan mendapati bahawa kepekatan klorin berkesan untuk mensterilkan spesies Burkholderia dan Burkholderia pseudomallei dalam julat sekurang-kurangnya 15 mg/L. Di samping itu, keberkesanan larutan klorin dapat dikekalkan jika digunakan dalam tempoh 2 hari persediaan tanpa kesan penting yang ditunjukkan daripada aspek masa perhubungan. Oleh itu, keberkesanan penyelesaian klorin untuk mensterilkan B. pseudomallei dan spesies Burkholderia berada dalam kepekatan klorin 15 mg / L yang disediakan dalam tempoh 48 jam dari tarikh persediaan.

Kata kunci: spesies Burkholderia, pensterilan, larutan klorin

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LIST OF ABBREVIATIONS

No.	Abbreviations	Full Name
1.	CDC	Center for Disease Conrol and Prevention
2.	*B.*	*Burkholderia*
3.	Spp.	Species
4.	pH	Potential of Hydrogen
5.	mg/L	An S. I. Unit stands for milligram per litre
6.	HTH	Calcium hypochlorite
7.	NaDCC	Sodium dichloroisocyanurate
8.	NaOCl	Sodium hypochlorite
9.	g	An S. I. Unit stands for gram
10.	L	An S. I. Unit stands for litre
11.	HOCl	Underchloric acid
12.	OCl	Hypochlorite ions
13.	HCl	Hydrochloric acid
14.	O	Atomair oxygen
15.	IHCM	Institute of Health and Community Medicine
16.	UHQ	Ultra high quality
17.	TSA	Tryptic soy agar
18.	NaCl	Sodium chloride
19.	mL	An S. I. Unit stands for millilitre
20.	µL	An S. I. Unit standds for microlitre
21.	OD	Optical Density
22.	CT	Contact Time
23.	SL	Shelf-Life
24.	DNA	Deoxynucleotide acid

1.0 INTRODUCTION

Burkholderia is a powerful bacterial genus of Gram-negative bacteria that possess versatility in genetic and metabolic, making these bacteria able to live through wide range of environmental settings both as free-living or host-associated microorganisms (Jackson & Coyne, 2018). The genus of *Burkholderia* is placed under the genera of betaproteobacteria (Liu *et al.*, 2014) and had been categorises into 2 groups: beneficial and pathogenic. *Burkholderia pseudomallei* is well-known as the causative agent for melioidosis which affects humans and animals. Due to the high virulence characteristics (such as resistance towards antibiotics and ability in avoiding human body defense system) in the microbe and the fact that the microbe can be transmitted via aerosolization (A. Jilani *et al.*, 2016), Center for Disease Control and Prevention (CDC) has categorised *B. pseudomallei* as a category B select agent (as cited by Hasselbring, Schell, & Patel, 2011). The fact that this microbe has been categorised as a potential bioterrorism agent has attracted researchers to have more detailed studies on the bacteria. The increasing interest among researchers for this bacteria has raised concerns for biosafety procedures and hence, this study for cost effective and efficient sterilization method is important to ensure biosafety for the reseachers and the environment.

1.1 Problem Statement

Due to its highly infectious characteristics, it is important to find ways to effectively sterilizes the microorganism. The most common method of sterilization is using chlorine, which is known for the effectiveness of disinfecting wide scope of microorganisms. Apart from that, chlorine is also considered to be one of the cheapest disinfectants and easily available in the market. Currently, chlorine solution is commonly used to disinfect

laboratory apparatus prior to be autoclaved for a more complete sterilization. The actual effectiveness for *B. pseudomallei* using the said method is not known.

1.2 Study Objective

The general objective of this study is to evaluate the effectiveness of chlorine solution in sterilizing *Burkholderia* species in laboratory.

The specific objectives of this study are stated as follows:

1. To compare the effectiveness of various concentrations of chlorine solutions on *Burkholderia* species and *B. pseudomallei*.
2. To study the effectiveness of chlorine solution with the different contact time with *Burkholderia* species and *B. pseudomallei*.
3. To study the shelf-life of chlorine solution and its effectiveness on *Burkholderia* species and *B. pseudomallei*.

2.0 LITERATURE REVIEW

2.1 *Burkholderia* Species

2.1.1 Introduction to *Burkholderia* Species

Burkholderia species is a bacterial genus of motile bacilli (Choh *et al.,* 2013), Gram-negtive soil and water-borne bacteria (Choh *et al.*, 2013; Jackson & Coyne, 2018), grouped under the genera of betaproteobacteria (Liu *et al.,* 2014). The genus *Burkholderia* has been created in the year of 1992, when Yabuuchi and coworkers (Yabuuchi *et al.,* 1992) reclassified *Pseudomonas* species belonging to rRNA group II. Since the reclassification, the genus of *Burkholderia* has undergone a lot of changes, such as increased of total species to over 30 species (Salles, van Veen, & van Elsas, 2004).

Burkholderia spp. are considered as multifaceted bacteria (wide variety in terms of genetic and metabolic versatility), which enable the bacterial genus to exist in broad scope of environmental niches, such as free-living or host-associated bacteria (Jackson & Coyne, 2018). The wide diversity of *Burkholderia* spp. also resulted in categorising of the genus into 2 groups: beneficial and pathogenic.

2.1.2 Beneficial species of *Burkholderia* Species

Beneficial species of *Burkholderia* aids a lot in plant growth, such as destruction of pest organisms, fixation of nitrogen in soil, increase disease resistance in plants and even contribution towards better water management (as cited by Compant *et al.*, 2008; Stoyanova *et al.*, 2007) and bioremediation (Salles, van Veen, & van Elsas, 2004; Stoyanova *et al.,* 2007). One example of beneficial *Burkholderia* spp. that can be deduced is *Burkholderia tuberum* strains specifically STM678 and DUS833 which are found by

Elliot (2007) (as cited by Compant *et al.*, 2008), showed that the strains are able to bring *Cyclopia* species and wide varieties of papillionoid legumes tribes to nodulate.

2.1.3 Pathogenic species of *Burkholderia* Species

The pathogenic strains of *Burkholderia* spp. are more well-known for the disease affecting human and animals than plants.

There are some infections in plant that is caused by *Burkholderia* spp. One of the examples is the onion rot caused by infection in onion leaves and bulbs by *B. cepacia*, as recorded by Burkholder W. H. in 1950 (as cited by Aw, 2014). Another *Burkholderia* infection is bacterial wilt in plant species which caused by *B. caryophylli*, as recorded by Furuya *et al.* in 2000 (as cited by Compant *et al.*, 2008; Aw, 2014). *B. plantarii* and *B. glumae* have caused wilting symptoms in many plants species and seedlings and grain rot in rice (Compant *et al.*, 2008). Compant and the colleagues (2008) also stated that *B. andropogonis* is capable of infecting more than 52 plant species.

The infections caused by these species towards animals, particularly human are harder to treat because of the species' properties of being resistant towards mutiple antibiotics, able to form biofilms and able to establish intracellular and chronic infections (Choh *et al.*, 2013). Example are like *Burkholderia mallei, Burkholderia cepacia* complex and *Burkholderia pseudomallei*. *B. mallei* has caused disease called glanders in humans and horses while *B. pseudomallei* has caused melioidosis in human and animals. *B. cepacia* complex is known for the complexity of the bacterial's genetic and lethality towards patients with cystic fibrosis (Choh *et al.*, 2013).

There are also other *Burkholderia* species that also cause disease, but the infections are very rare, such as *B. thailandensis* (Haraga *et al.*, 2008) and *B. multivorans* (Zahariadis, Levy, & Burns, 2003).

2.2 *Burkholderia ubonensis*

Burkholderia ubonensis is an opportunistic bacterium found environmentally (usually in soil) that generally is listed as a strain that bring nonfatal infections within healthy individuals (Prince *et al.*, 2013). Genetically, *B. ubonensis* is categorised under *B. cepacia* complex (Bcc) but commonly mistaken as *B. pseudomallei* due to the fact that these two strains shared similar environmental niche and morphology (Prince *et al.*, 2013). Unfortunately, there is not much study done on *B. ubonensis*.

A study led by Prince and the coworkers in the year of 2013 found out that *B. ubonensis* could be useful to be utilised as a biocontrol control agent towards *B. pseudomallei*, especially in melioidosis endemic regions because these two strains possess antagonistic property to one another.

2.3 *Burkholderia thailandensis*

Burkholderia thailandensis is a bacterium that closely-related to *B. pseudomallei* that rarely cause infections to human (Haraga *et al.*, 2008). The reason behind this is because while these two strains do share similar virulence factors and genomic with one another, *B. thailandensis* lacks the TTS1 gene that renders *B. pseudomallei* the ability to cause disease in humans and animals (Chang *et al.*, 2017). Another discrete difference between these two species that distinguished from one another is that *B. thailandensis* is able to assimilate L-arabinose, unlike *B. pseudomallei* which is lacking of the whole operon of arabinose-assimilation (Haraga *et al.*, 2008; Chang *et al.*, 2017).

Despite of the fact that *B. thailandensis* rarely causes disease, a case study made by a team of Chinese researchers led by Cheng K. in the year 2017 revealed a different story: a 67-year-old man of Chongqing is being hospitalised for severe fever, productive cough with

white sputum and shortness of breath. Despite being given antimicrobial drug from a local clinic and 6-day treatment in hospital, his condition did not improved; because of his family longed to be close to him, he died 2 days after being discharged from hospital. There were also 2 case reports of *B. thailandensis* in Thailand and United States, describing soft tissue infection and pneumonia with sepsis (Chang *et al.*, 2017).

2.4 *Burkholderia pseudomallei*

2.4.1 Introduction to *Burkholderia pseudomallei*

Burkholderia pseudomallei which is a Gram-negative (A. Jilani *et al.*, 2016), facultative anaerobic, motile (Larsen, 2009) and saprophytic bacterium which has resistance towards a number of antibiotics (Podin *et al.*, 2014), is the causative organism of melioidosis. The species is actively found in moist soils and surface waters of regions with tropical and subtropical climate (Stone *et al.*, 2014). The species infects animals and human, with main reservoir from contaminated soil and water (Gilad, Schwatz, & Amsalem, 2007). Since the species is soil and water-borne, it explains why there were high recorded cases of melioidosis during rainy seasons (Stone *et al.*, 2004). Another reason that considers *B. pseudomallei* as a threat is because the symptoms present in *B. pseudomallei* infected patients are very similar to other common respiratory infections that often caused misdiagnosis of infections (Aw, 2014). Aw (2014) also stated that *B. pseudomallei* often get misdiagnosed as *B. cepacia*, *Pseudomonas aeruginosa, Pseudomonas fluorescens* and *Chromobacterium* sp due to unexperienced laboratory workers or limited validated diagnostic equipments. Another research done by Howard and Inglis (2003a) claimed that atypical colony morphology of some *B. pseudomallei* strains and presence of species that are similar or closely related to *Burkholderia* species caused further complexity in identification of *Burkholderia* species.

2.4.2 Melioidosis due to *Burkholderia pseudomallei*

The bacterial strain of *B. pseudomallei* is viewed more as a potential bioterrorism bacterial agent within the circle of medical and laboratory personnel (Gilad, Schwatz, & Amsalem, 2007). Human acquired melioidosis via 3 main entry pathways: inhalation, inoculation (via skin) or ingestion with highest cases recorded during rainy seasons in certain countries (Stone *et al.*, 2014), while cases have been found to be not correlated to rainfall in Sarawak (Mohan et al., 2017)

There are a number of places where melioidosis is found endemic, such as Southeast Asia and Oceania, especially in Northern Australia, Thailand, Singapore, Vietnam, Malaysia, Burma and Bangladesh (Gilad, Schwatz, & Amsalem, 2007; A. Jilani *et al.*, 2016), whereas some places recorded melioidosis as an emerging infectious disease, such as in South America (specifically Puerto Rico), Africa and India (Stone *et al.*, 2014).

2.4.3 *Burkholderia pseudomallei* and Melioidosis in Sarawak, Malaysia

A study conducted by Podin *et al.* (2014) showed that *B. pseudomallei* isolates in central Sarawak were found to be susceptible towards aminoglycoside (gentamicin) and existed in unusually high isolation rate. Another study conducted by Mohan *et. al.* (2017) recorded that Sarawak has very high cases of paediatric melioidosis, compared with world statistics of reported cases (approximately less than 5%).

2.5 *Burkholderia pseudomallei* as Potential Bioterrorism Weapon

Cases recorded in Thailand showed that *B. pseudomallei* has high infectivity and still can be lethal even when the patients underwent antibiotic therapy. As such, Thailand has listed *B. pseudomallei* as a bio-threat agent (Hasselbring, Schell, & Patel, 2011). This is because *B. pseudomallei* is resistant to a number of antibiotics and recovering patients will be

needing prolonged eradication therapy phase of treatment (Stone *et al.*, 2014). However, recurrent cases of melioidosis are also common and to add things worse, there is no vaccine available for melioidosis (Stone, DeShazer, Brett, & Burtnick, 2014). Due to this, Centre for Disease Control and Prevention (CDC) has categorised *B. pseudomallei* a category B select agent (Hasselbring, Schell, & Patel, 2011).

Some factors that contribute into increased infectivity and lethality of *B. pseudomallei* when compared with other bacterial pathogens are the exopolysaccharide capsule, lipopolysaccharide O antigen, type IV pili and type II, III and VI secretion systems (Hasselbring, Schell, & Patel, 2011). The capsules of *B. pseudomallei* preventing the host complement to opsonophagocytosise the bacterium, and thus ensuring survival of the bacterium (Stone *et al.*, 2014).

Some studies recorded the virulence mechanism of *B. pseudomallei* and found out that the bacterium not only is able to escape from phagocytic vacuole, but can freely reproduce in host's cell cytosol and cause the host cells to fuse into multinucleated giant cells in the end (Hasselbring, Schell, & Patel, 2011).

2.6 Sterilization

Based on Laboratory Manual of Biosafety in Microbiology and Biomedical Laboratories (U.S. Department of Health and Human Services, 2009) sterilization is a procedure of killing off all microorganism, including bacterial endospores. An item, device or solution will only be considered sterile when the probability of a microorganism (all living cells and viruses) to survive is less than one in one million (10^{-6}), passing the sterile assurance level. The usage of autoclave or dry heat and radiation (example: ultraviolet rays) are some of the examples of various methods of sterilization in laboratory (University of Colorado Boulder,

2008). But in this study, the proposed method of sterilization being evaluated is chlorine solution, which is known as general disinfectant in laboratories.

2.7 Chlorine Solution

2.7.1 Introduction to Chlorine

Chlorine is very effective against huge varieties of bacteria and viruses (Earth Tech Inc., 2005). When it is compared as a disinfectant, chlorine is more stable than chlorine dioxide but less effective in terms of disinfection than ozone. The process of mixing chlorine into a substance particularly into water reservoir for disinfection purpose is called chlorination. In general, chlorine is able to produce necessary residual protection and hence, an excellent disinfectant for water and surface disinfection (Earth Tech Inc., 2005). Apart from that, chlorination is also a very economical process (Earth Tech Inc., 2005).

2.7.2 Chlorine in Other Researches

Chlorine is also being used in other researches in terms of disinfection. Some examples have been tabulated into Table 1:

Table 1: Comparison of Chlorine usage across researches

Researchers (Years)	Research done	Target(s)	Concentration of Chlorine Solution Used
W. McGlynn (*n.d*)	Guidelines for the Use of Chlorine Bleach as a Sanitizer in Food Processing Operations	Food processing equipment and food contact articles	Chlorine available not exceeding 200 mg/L
		Raw fruits and vegetables (general)	Total chlorine lower than 200 mg/L followed by thorough rinsing
		Water (for processing)	Residual chlorine not exceeding 0.5 mg/L
Iqbal, Luberk-Schriker, Wells, Wolfe, & Lantagne (2016)	Shelf-Life of Chlorine Solutions Recommended in Ebola Virus Disease Response	Washing living things during Ebola outbreaks (e.g. hands, body parts)	500 mg/L
		Washing non-living things during Ebola outbreaks (e.g. surfaces, personal protective equipment)	5 000 mg/L

2.7.3 Contact Time between Chlorine Solution and Bacterial Isolates

According to Oram (2014), the contact time depends on the chlorine concentration present, type of pathogens involved, pH of the solution and the temperature of the solution, with the

fact that the contact time must increase under condition of low temperature or high alkalinity of the solution. Calculations done by Oram himself in 'Chlorination of Drinking Water' published by Water Research Centre, found that chlorine residual of 0.5 mg/L needs 30 minutes of contact time while chlorine residual of 0.3 mg/L needs 50 minutes, under condition of pH level at 7.5 and temperature at 5.5 ℃.

2.7.4 Shelf-life of Chlorine Solution

According to Iqbal and the colleagues (2016), chlorine solutions is very sensitive to pH, temperature and concentration and degraded highly depending on the 3 components. However, the chlorine solutions used by the team are basically made up of 3 type of chlorine solutions: powdered calcium hypochlorite (HTH), granular sodium dichloroisocyanurate (NaDCC) and liquid sodium hypochlorite (NaOCl) with pH ranging from 5 to 11. Both of HTH and NaDCC are a high-concentration effervescent powder but HTH is known for the capability to clog pipes; on the other hand, NaOCl is a solution easily being made locally or purchased industrially. Hence, the shelf-life of the chlorine solution that evaluated by the team was defined as days until the concentration of the chlorine solution dropped to more than 90% of the initial concentration in laboratory conditions. The temperature of the environment is kept in range of 25 to 35 ℃. The results were recorded in Table 2:

Table 2: Shelf-life of chlorine solutions based on reseach conducted by Iqbal *et al.* (2016)

Type of Chlorine Solution used	pH	Shelf-life
Neutralized-NaOCl	7	Few hours
NaDCC	6	2 days
Generated NaOCl	9	6 days
HTH	9-11	More than 30 days
Stabilized NaOCl	9-11	More than 30 days

2.7.5 Advantages and Disadvantages of Chlorine

Based on a Laboratory Manual written by Earth Tech Inc. (2005), the principle advantages of chlorine are the following:

- Chlorine is highly effective against wide varieties of bacteria and viruses.
- Chlorine produces necessary residuals that give protection from bacteria and viruses to survive in the system when used in lower concentration.
- Chlorine is an economical disinfectant and available in most laboratories.

Meanwhile, the principle disadvantages of chlorine are listed as:

- Chlorine is not effective against protozoan oocysts, such as *Cryptosporidium*.
- Chlorine reacts with natural organic matter to form halogenated by-products which considered as toxic to human health.
- High doses of chlorine in water chlorination can cause taste and odour problems.